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Silymarin attenuates benzo(a)pyrene induced toxicity by mitigating ROS production, DNA damage and calcium mediated apoptosis in peripheral blood mononuclear cells (PBMC)

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ABSTRACT

Benzo(a)pyrene (B(a)P), which is the most studied member of PAH family is released into the environment (air, water and soil) from natural and man-made sources including industrial and automobile exhaust fumes. Since B(a)P is an omnipresent environmental pollutant and is believed to be a risk factor for human chemical carcinogenesis, it is important to identify potent naturally occurring/synthetic agents that could modulate B(a)P-induced toxicity. The present study explores the effect of the flavonoid silymarin (2.4 mg/ml) in counteracting the toxicity of B(a)P (1 μM) in PBMC. Flourimetry and Confocal Laser Scanning Microscopy results showed that silymarin reduces the B(a)P induced ROS production and DNA damage. Atomic Absorption Spectroscopy analysis and fluorescent microscopic pictures proved that silymarin reduces the increased intracellular calcium and apoptosis induction during B(a)P treatment. Furthermore, silymarin did not show any inhibition for CYP1B1 activity at transcriptional level by semiquantitative RT PCR but it affects the catalytic activity of Phase I CYP1A1/CYP1B1 enzyme (EROD assay) during B(a)P treatment. The findings reveal that silymarin possesses substantial protective effect against B(a)P induced DNA damage and calcium mediated apoptosis by inhibiting the catalytic activity of CYP1B1 and maintaining the intracellular calcium dysregulation; hence, it could be considered as a potential protective agent for environmental contaminant induced immunotoxicity.

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1. Introduction

The polycyclic aromatic hydrocarbon (PAH), benzo(*a*)pyrene (B[*a*]P), is an ubiquitous environmental pollutant, which is released into the environment (air, water and soil) from natural and man-made sources (Dutta et al., 2010). In cells, B(a)P binds to and activates a ligand-dependent transcription factor termed aryl hydrocarbon receptor (AhR). The ligand-activated AhR–ARNT complexes interact with specific promoter elements (termed xenobiotic response elements; XREs), which induce the transcription of a number of genes involved in the metabolism of B(a)P, including CYP1A1, CYP1A2, and CYP1B1 (Hankinson, 1995). CYP1A1 and CYP1B1 activates B[*a*]P to B[*a*]P-7,8-oxide, which through hydration by epoxide hydrolase, is metabolized to (±)-B[*a*]P-trans-7,8-dihydrodiol (B[*a*]P-7,8-DHD). B[*a*]P-7,8-DHD may then serve as a substrate for a second CYP-dependent oxidation reaction, generating the ultimate carcinogenic metabolite B[*a*]P-7,8-dihydroxy-9,10-epoxide (BPDE).These BPDE binds to DNA and generates bulky DNA adducts primarily at deoxyguanosine. The glutathione S-transferases (GSTs) which is the major group of conjugative enzymes in phase II detoxification deals with the cellular damage caused by BPDE. Hence the dynamic competition between the activation of B(a)P by CYPs and the opposing detoxification of BPDE by GSTs is a critical determinant of the genotoxicity of B(a)P.

Since B(a)P is an omnipresent environmental pollutant and is believed to be a risk factor for human chemical carcinogenesis, it is important to identify potent naturally occurring/synthetic agents that could modulate B(a)P-induced toxicity. The present study explores the protective effect of silymarin in PBMC, which is

Abbreviations: AAS, Atomic Absorption Spectroscopy; AhR, Aryl hydrocarbon receptor; ANF, Alpha napthoflavone; B[*a*]P-7,8-DHD, (±)-B[*a*]P-*trans*-7,8-dihydrodiol; B[a]P, Benzo[a]pyrene; BPDE, B[*a*]P-7,8-dihydroxy-9,10-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene; CLSM, Confocal laser scanning microscopy; CYP, Cytochrome; DCFH-DA, Dichlorflourescein diacetate; EROD, Ethoxyresorufin deethylase activity; GSTs, Glutathione S-transferases; H_2O_2, Hydrogen peroxide; MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; $O_2^{\bullet}-$, Superoxide; $OH^{\bullet}$, Hydroxyl radical; PAH, Polycyclic aromatic hydrocarbons; PBMC, Peripheral Blood Mononuclear Cells; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; XREs, Xenobiotic response element

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used as the predictive model for screening the effect of drugs, because of its suitability for assessing the immune activation and immunomodulation (Saurabh et al., 2010). Our earlier studies have shown that silymarin protects the blood cells such as erythrocytes against B(a)P (Kiruthiga et al., 2007a) and H_2O_2 (Kiruthiga et al., 2007b) induced toxicity and PBMC against B(a)P induced oxidative stress (Kiruthiga et al., 2010). The protective potential of silymarin against B(a)P in PBMC was studied by evaluating the rate of apoptosis and ROS production, along with the evaluation of Phase I and Phase II detoxification enzymes which are involved in the metabolism of B(a)P. The role of intracellular calcium in B(a)P mediated apoptosis and the role of silymarin in regulation of calcium was also elucidated. Our results demonstrate that silymarin has a remarkable protective potential against B(a)P induced oxidative stress damages and immunotoxicity in human PBMC. The effect of silymarin was further compared with the positive control alpha napthoflavone (ANF), which is a weak aryl hydrocarbon receptor agonist.

2. Materials and methods

B[a]P was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Silymarin, the commercially available plant flavonoid was purchased from Ranbaxy Laboratory Limited, Manipur, India. The other chemicals and reagents used were of analytical grade

2.1. Blood collection

In accordance with Helsinki Declaration, the design and execution of the experiment were thoroughly explained to the participants. Venous blood was collected by venipuncture from each human healthy volunteer into EDTA containing tubes.

2.2. Peripheral Blood Mononuclear Cells (PBMC) isolation

Peripheral Blood Mononuclear Cells (PBMC) were separated by density gradient centrifugation at 400 g using Lymphocyte Separation Medium (Laboratories Eurobio, France).

2.3. Checking for PBMC viability by trypan blue exclusion

Cell viability was checked by trypan blue and the cell suspension of $>95\%$ viability were adjusted to a concentration of 1×10^6 cells per ml for further experiments. The cells were divided into six groups. **Group I** vehicle treated: treated with $<0.2\%$ DMSO; **Group II** B(a)P treated: treated with 1 μM of B(a)P (adopted from Wilms et al. (2005)); **Group III** B(a)P+silymarin: treated with 1 μM of B(a)P+2.4 mg/ml of Silymarin [21]; **Group IV** silymarin alone: treated with 2.4 mg/ml of Silymarin; **Group V** B(a)P+α-napthoflavone: treated with 1 μM of B(a)+10 μM α-naphthoflavone; **Group VI** α-naphthoflavone alone: treated with 10 μM α-Naphthoflavone alone. The cells were exposed to silymarin/B(a)P for 18 h in a CO_2 incubator (5%) and then assessed for the various parameters.

2.4. Assessment of metabolic activity of the cells by MTT assay

Cell viability was assessed by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. PBMC were treated with silymarin/B(a)P, washed with PBS and treated with fresh medium containing MTT (2 mg/ml) and were incubated for 3 h at 37 °C in dark. The blue MTT formazan product formed was determined at 570 nm in a spectrophotometer. The results are represented as percent of viable cells compared with the vehicle control.

2.5. Morphological assessment of cells

After the experimental period, PBMC was washed two–three times with PBS to remove any debris and the cells were mounted in a glass slide and viewed in a light microscope to visualize the morphology.

2.6. Morphological assessment of apoptosis by fluorescent microscopy

Apoptosis was studied morphologically using fluorescent dyes (ethidium bromide/acridine orange) that intercalate DNA by the method of (Duke and Cohen, 1992). Acridine orange stains DNA bright green, allowing visualization of nuclear chromatic pattern. Apoptotic cells have condensed chromatin that is uniformly stained. Ethidium bromide stains DNA orange, but is excluded by viable cells and it can be viewed by fluorescent microscopy.

2.7. Detection of DNA damage by alkaline single cell electrophoresis (alkaline comet assay)

Comet assay was assessed by the method of (Falcioni et al., 2010) to evaluate DNA strand breaks in individual cells. Imaging was performed using AutoComet™ scoring software to determine tail length, tail intensity and tail moment (TL, TI and TM)—all parameters correlated with the degree of DNA damage in the single cells.

2.8. Detection of intracellular ROS

Formation of intracellular ROS was measured by using DCFH-DA dye as described by (Royall and Ischiropoulos, 1993) and the images were collected in a Confocal Laser Scanning Microscopy (Model: LSM 710, Carl Zeiss, Germany).

2.9. Measurement of nitrite content

Nitric oxide production was measured in terms of nitrite using standard procedures (Granger et al., 1996) and the nitrite content was calculated using a standard curve for sodium nitrite (10–100 μM) and is expressed in μM. After incubation, the cell suspensions were washed with PBS to remove silymarin, B(a)P and lysed using lysis buffer. Cytosol obtained was centrifuged to remove debris and the supernatant was mixed with Griess reagent to measure the nitrite production.

2.10. Measurement of intracellular calcium

The concentration of intracellular Ca^{2+} was measured by using Atomic Absorption Spectroscopy (AAS) (VARIAN Model SPECTRAA 220).

2.11. EROD assay

EROD activity was assayed by the method of Klotz et al. (1984) is reported as the rate of resorufin formation, which is proportional to the amount of CYP1A1/CYP1B1 enzymes present. Utilization of substrate ethoxyresorufin by CYP1A1/CYP1B1 forms the resorufin in the presence of NADPH, which was observed by reading the absorbance at 572 nm for 3 min using spectrophotometry. Formation of resorufin was calculated from extinction coefficient of resorufin 73 mM/cm.

2.12. RNA isolation and analysis

Total RNAs were extracted from PBMC (5×10^6) by the TRIzol method and subjected to reverse transcription-semi-quantitative polymerase chain reaction using the Superscript III kit (Invitrogen) according to the manufacturer's instructions. After cDNA synthesis, PCR was performed using gene specific primers CYP1A1-(F-5′TGGACATGACCCCCATCTAT3′ R-5′AGGGTCCTGGTTTGGCTAGT3′), CYP1B1 (F-5′CCTGACCAGCAGAGTGATGA3′ R-5′ AGCTCCTGCATAGCCCACTA3′), ß-actin (F-5′GATGAGATTGGCATGGCTTT3′ R-5′ CACCTTCACCGTTCCAGTTT3′) shown in Table 1. Primers were designed and checked for specificity by BLAST search, the purity of the PCR products and the specificity of the reaction were checked by gel electrophoresis analysis.

2.13. Assay of the phase II enzyme GST

GST activity was measured based on the rate of formation of GSH conjugate with CDNB. The activity was assayed using spectrophotometry and the enzyme units were calculated using extinction coefficient of GSH (9.6 M^{-1} cm^{-1}). GST was assayed by (Habig et al., 1974) method and the activity was expressed as 1 mM of GSH decomposed/min/mg of protein using an extinction coefficient of GSH.

Table 1
Gene specific primers used for RT-PCR analysis.

Gene	Primer sense	Primer antisense
β-actin	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT
CYP1A1	TGGACATGACCCCCATCTAT	AGGGTCCTGGTTTGGCTAGT
CYP1B1	CCTGACCAGCAGAGTGATGA	AGCTCCTGCATAGCCCACTA

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